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Genomic Markers of Ancient Anaerobic Microbial Pathways: Sulfate Reduction, Methanogenesis, and Methane Oxidation

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Genomic markers for anaerobic microbial processes in marine sediments—sulfate reduction, methanogenesis, and anaerobic methane oxidation—reveal the structure of sulfate-reducing, methanogenic, and methane-oxidizing microbial communities (including uncultured members); they allow inferences about the evolution of these ancient microbial pathways; and they open genomic windows into extreme microbial habitats, such as deep subsurface sediments and hydrothermal vents, that are analogs for the early Earth and for extraterrestrial microbiota.

Sulfate reduction and methanogenesis are two terminal anaerobic bioremineralization pathways that convert low-molecular-weight products of other bacterial processes (degradation of polymers, fermentation) to CO_2 and methane. Sulfate-reducing bacteria are physiologically and phylogenetically highly diverse (Castro *et al.*, 2000; Widdel and Bak, 1992); they oxidize a wide variety of low-molecular-weight compounds (short-chain fatty acids, alcohols, alkanes, aromatic compounds, acetate) to CO_2. In marine sediments, the range of sulfate-reducing bacteria is limited by sulfate availability. When sulfate is depleted, methanogenic archaea become the dominant anaerobic microbial population. Autotrophic methanogens utilize hydrogen as energy source for the reduction of CO_2 to methane; specialized genera of methanogens are also capable of inter- and intramolecular disproportionation of C_1 and C_2 carbon compounds (methanol, methylamines, acetate) to methane and CO_2 (Boone *et al.*, 1993). Where methane and sulfate coexist (for example, at the interface of sulfate-reducing and methanogenic sediment layers, or at marine methane seeps and vents), sulfate-dependent anaerobic methane oxidation takes place; methane of biogenic origin is oxidized to CO_2 with sulfate as the terminal electron acceptor (Valentine and Reeburgh, 2000). As proposed originally on the basis of biogeochemical field data and thermodynamic considerations (Hoehler *et al.*, 1994), anaerobic methane oxidation is carried out by syntrophic consortia of methanotrophic archaea and sulfate-reducing bacteria, in which the sulfate-reducing partner catalyzes the electron transfer from methane to sulfate and assimilates a portion of the methane oxidation products (Boetius *et al.*, 2000; Orphan *et al.*, 2001b). Methanotrophic archaea of different phylogenetic affiliation can form dense, highly ordered clusters with sulfate-reducing syntrophs, or may occur in less tight associations (Orphan *et al.*, 2002).

The sulfate-reducing, methanogenic, and methane-oxidizing microbial populations that are found in anaerobic marine microbial ecosystems today are the modern descendants of ancient microbiota whose isotopic imprints are pervasive in the carbon- and sulfur-isotopic record, from the present back to the Archaean-Proterozoic transition (Knoll and Canfield, 1998). The only oxidants that these pathways require are CO_2 or carbonate, and sulfate, which existed before the photosynthetic oxygenation of the Earth's bio-

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sphere and the appearance of free oxygen in the atmosphere and the marine water column. Isotopic evidence for widely expressed microbial sulfate reduction, in the form of ^{34}S-depleted sedimentary sulfides, goes back to the middle and early Proterozoic, 2.2 to 2.3 billion years ago (Canfield *et al.*, 2000). The mineralization of organic matter by methanogenesis, followed by methane oxidation, may even predate the onset of sulfate reduction. The carbon isotopic imprint of this process, in the form of highly ^{13}C-depleted kerogen ($\delta^{13}C \geq -60‰$), is found in late Archaean and early Proterozoic kerogens, 2.8 billion years old (Strauss and Moore, 1992). This isotopic record was originally interpreted as evidence for widespread aerobic methane oxidation (Hayes, 1994). Anaerobic methane oxidation is more likely, since evidence for the stepwise and pervasive oxygenation of the proterozoic biosphere begins to appear only at a later time, about 2.2 billion years ago (Des Marais *et al.*, 1992).

Key Genes for Sulfate Reduction and Anaerobic Methane Cycling

The antiquity and evolutionary significance of these microbial pathways is shown in the high degree of phylogenetic conservation of their key genes and key enzymes. In sulfate-reducing prokaryotes, the *aps* gene codes for the key enzyme adenosine-5′-phosphosulfate reductase, which catalyzes the activation and subsequent reduction of sulfate to sulfite (Friedrich, 2002). A second key gene of dissimilatory sulfate reduction, *dsrAB*, codes for the alpha and beta subunits of the enzyme dissimilatory sulfite reductase, which catalyzes the reduction of sulfite to sulfide (Wagner *et al.*, 1998). The *dsrAB* and *aps* genes are phylogenetically conserved in several deeply branching phyla of bacterial and archaeal sulfate reducers. When specific gene transfer events are taken into account, the *dsrAB* and *aps* genes allow a simultaneous phylogenetic and metabolic identification of sulfate-reducing prokaryotes (Klein *et al.*, 2001; Friedrich, 2002).

Coenzyme M methyl reductase is the key enzyme of methanogenesis; it catalyzes the terminal and highly exergonic step of the methanogenesis pathway, the reduction and release of the coenzyme-M-bound methyl group as free methane. The Coenzyme M methyl reductase gene (*mrcA*) is found in methanogenic archaea; it is sufficiently conserved and consistent with 16S rRNA phylogenies to allow the identification of methanogenic archaeal lineages in environmental samples (Springer *et al.*, 1995; Lueders *et al.*, 2001; Ramakrishnan *et al.*, 2001). At present it is not known whether anaerobic methane-oxidizing archaea are using a version of this enzyme for the activation and reoxidation of methane. If anaerobic methane oxidation by archaea could proceed through a reversal of classical methanogenesis pathways, the Coenzyme M methyl reductase reaction would be the most difficult and energy-demanding step to reverse (Hoehler and Alperin, 1996). Current full-genome sequencing efforts using purified ANME-1 and ANME-2 archaea from environmental samples are testing whether the genomes of these methanotrophic archaea carry coenzyme M methyl reductase genes (Orphan *et al.*, 2002).

The Guaymas Basin Hydrothermal Vent Sites as Model System

To search for deeply branching and (possibly) ancestral representatives of sulfate-reducing, methanogenic, and methane-oxidizing microorganisms and their key genes in modern environments, we focus on hydrothermal vent habitats. Hydrothermal vents represent some of the earliest and best protected microbial habitats that may have survived repeated environmental disturbances in the surface biosphere; vents can in principle occur on every planet with oceans and active plate tectonics or volcanism. On Earth, hydrothermal vents sustain complex microbial ecosystems that utilize inorganic energy sources (such as sulfide, hydrogen, and reduced metals) and geothermal sources of carbon (such as methane, CO_2, and geothermally synthesized low-molecular-weight organic compounds) (Kelley *et al.*, 2002). The hydrothermally active sediments of the Guaymas Basin (Gulf of California, Mexico) provide a relatively well-studied model system for the complexity of the microbial communities that are involved in sulfate reduction, methanogenesis, and methane oxidation. Cultivations, lipid biomarker analyses, 16S rRNA, and functional gene sequencing are beginning to reveal unusually complex microbial communities that include sulfate-reducing prokaryotes, methanogenic archaea, and anaerobic methanotrophic archaea and their sulfate-reducing syntrophs (Fig. 1). Specifically, results of the Guaymas Basin survey (Teske *et al.*, 2002; Dhillon *et al.*, unpubl.) will also help to identify novel sulfate-reducing, methanogenic, and methane-oxidizing microorganisms in deep subsurface sediments, where these processes are predominant (D'Hondt *et al.*, 2002). These anoxic environments represent analogs to subsurface life under extraterrestrial conditions in which an inhospitable surface environment might have driven microbial life underground or never allowed its evolution within a phototrophic, oxygenated biosphere.

Guaymas Basin Microbial Communities

Sulfate-reducing bacteria and archaea are dominant terminal oxidizers of organic matter in the Guaymas Basin, as shown by high rates of sulfate reduction measured over wide temperature ranges up to about 100°C (Jorgensen *et al.*, 1990, 1992; Elsgaard *et al.*, 1994; Weber and Jorgensen, 2002). Hyperthermophilic, autotrophic, or mixotrophic archaea of the genus *Archaeoglobus* were found by cultivation (Burggraf *et al.*, 1990) and 16S rRNA sequencing

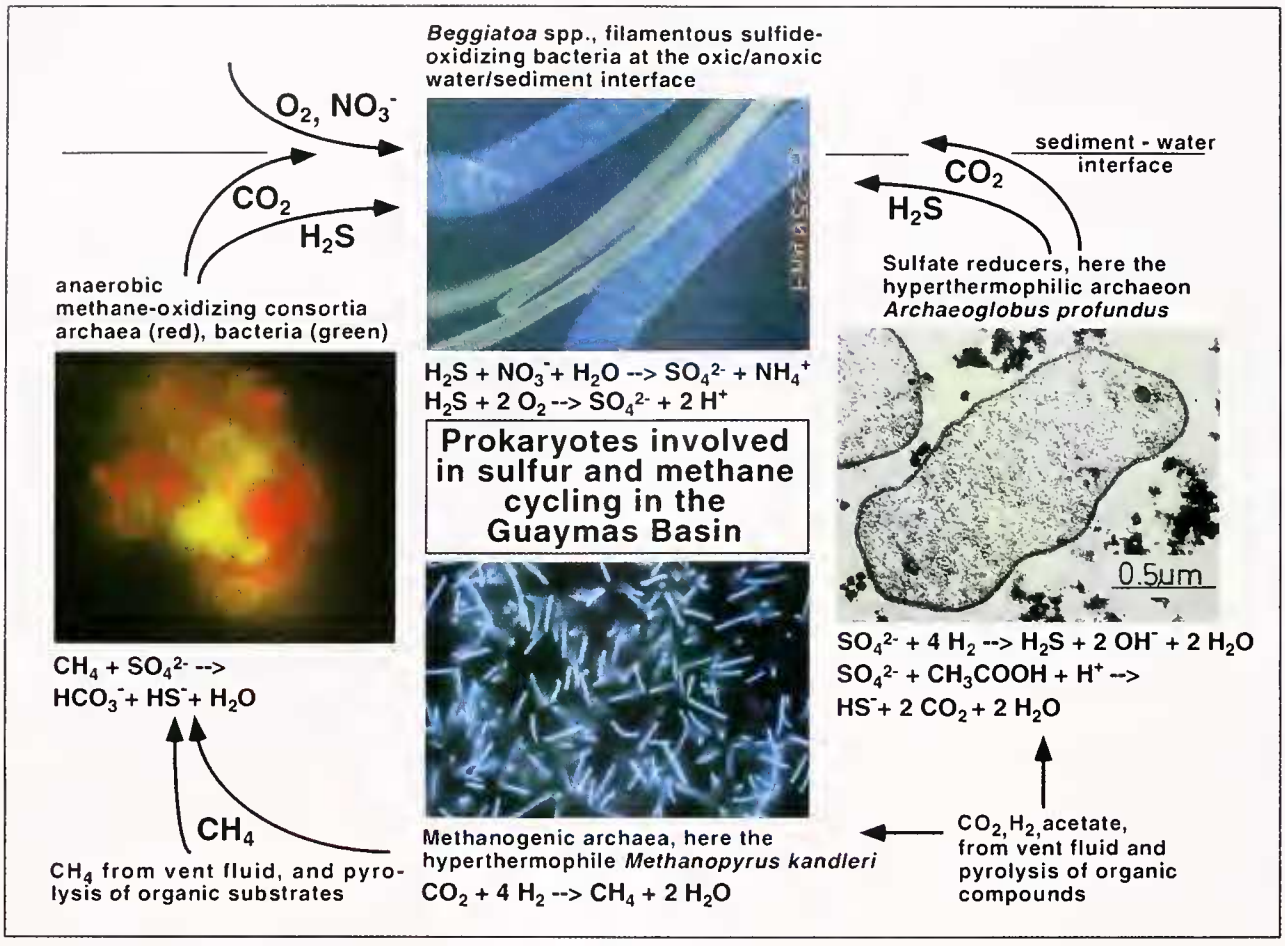

Figure 1. Simplified scheme of microorganisms and their reactions (sulfate reduction, methanogenesis, methane oxidation, sulfide oxidation) in the methane and sulfur cycles in the Guaymas Basin hydrothermal vents. Clockwise, sulfate-reducing bacteria and archaea (*Archaeoglobus profundus*), methanogenic archaea (*Methanopyrus kandleri*), methane-oxidizing consortia, and sulfide-oxidizing bacteria (*Beggiatoa* spp.). Fluorescence *in situ* hybridization image of anaerobic methane-oxidizing consortium, courtesy K. Knittel and A. Boetius (Max Planck Institute for Marine Microbiology, Bremen, Germany). The bacterial and archaeal species shown here are representatives of metabolically and phylogenetically diversified functional classes of prokaryotes. It has to be noted that sulfate reduction and sulfate-dependent methane oxidation are almost certainly uncoupled in the Guaymas sediments. The highly diversified sulfate-reducing prokaryotic community can oxidize a wide range of substrates (H_2 and acetate are just the simplest examples), whereas methane-oxidizing syntrophs are most likely restricted to methane oxidation intermediates provided by their archaeal partners.

(Teske *et al.*, 2002). Moderately thermophilic or mesophilic fatty acid oxidizing sulfate reducers have been cultured from Guaymas (Rueter *et al.*, 1994). Surveys with 16S rRNA detected predominantly members of the propionate-oxidizing, acetate-producing family Desulfobulbaceae (Teske *et al.*, 2002) and members of the acetate-oxidizing family Desulfobacteriaceae (Dhillon *et al.*, 2002). A molecular survey based on *dsrAB* genes revealed the existence of novel sulfate reducers in the Guaymas Basin that are not related to any cultured group of sulfate-reducing prokaryotes (Dhillon *et al.*, 2003).

Methanogenic archaea in the Guaymas Basin include hyperthermophilic, autotrophic methanogens of the genera *Methanococcus* (Jones *et al.*, 1989; Canganella and Jones, 1994) and *Methanopyrus* (Kurr *et al.*, 1991), and members of the formate-utilizing, mesophilic or moderately thermophilic family Methanomicrobiales (Teske *et al.*, 2002). Methane produced by these diverse methanogenic communities combines with the methane pool originating from pyrolysis of organic matter buried in the Guaymas sediments; the resulting methane concentrations in the Guaymas vent fluids are orders of magnitude higher than at non-sedimented, bare lava vent sites (Welhan, 1988).

Anaerobic methanotrophic communities in the Guaymas Basin include ANME-1 and ANME-2 archaea, as shown by 16S rRNA gene sequence analysis and ^{13}C-isotopic analysis

of diagnostic archaeal lipids (Teske *et al.*, 2002). The sulfate-reducing syntrophs in the Guaymas Basin sediments could not be identified unambiguously by 16S rRNA sequencing; their ^{13}C-depleted membrane lipids (mono- and dialkylglycerol ethers) indicate deeply branching bacteria or sulfate-reducing bacteria of the family Desulfosarcinales (Teske *et al.*, 2002). In classical ANME-2 consortia at Hydrate Ridge and Eel River Basin, the archaeal core was surrounded by an outer layer of sulfate-reducing bacteria of the family Desulfosarcinales (Boetius *et al.*, 2000; Orphan *et al.*, 2001a, b). In Guaymas Basin samples that yielded ANME-2 sequences, fluorescence *in situ* hybridization revealed a different structure; archaeal cells were intertwined with irregular lobes of syntrophic bacteria that did not hybridize with the probe for members of the Desulfosarcinales (Knittel *et al.*, 2002). A similar structure of ANME-2 archaea intertwined with unidentified bacteria has been observed in anaerobic methane-oxidizing consortia from Eel River Basin (Orphan *et al.*, 2002), and in the Haakon-Mosby Mud Volcano in the Norwegian Arctic Ocean (Knittel *et al.*, 2002).

Autotrophic sulfide-oxidizing bacteria of the genus *Beggiatoa* (Nelson *et al.*, 1989) that grow in dense mats on the sediment surface assimilate CO_2 from seawater and sediments; the latter CO_2 pool includes contributions from sulfate reduction and methane oxidation. The assimilation of methane oxidation products by sulfur-oxidizing bacteria appears to be highly variable. The ^{13}C isotopic signals of *Beggiatoa* biomass from hydrocarbon and methane seeps range from typical values of about $-20‰$ to $-30‰$ (Sassen *et al.*, 1993) to strong ^{13}C depletion indicative of assimilation of methane oxidation products (Paull *et al.*, 1992). The oxidation of sulfide and other sulfur intermediates (produced by sulfate-reducing bacteria) by *Beggiatoa* spp. depends on the availability of oxygen or nitrate as the terminal electron acceptor (McHatton *et al.*, 1996). Therefore, *Beggiatoa* spp. and other free-living and symbiotic sulfide-oxidizing bacteria that represent the basis of the food chain at hydrothermal vents could not survive in a strictly anoxic microbial habitat that does not receive molecular oxygen from the photosynthetic biosphere. In this way, the Guaymas Basin shows the caveats and limits of early-earth or astrobiological analogs. Also, sulfate reduction and methanogenesis in the Guaymas sediments are ultimately fuelled by high sedimentation of terrestrial organic matter and upper water column primary production; in other words, they depend on products of the oxygenated, photosynthetic biosphere.

Potential of Conserved Functional Genes for Genomics

Screening a microbial community for highly conserved key genes of sulfate reduction, methanogenesis, and methane oxidation results in a diversity census, with emphasis on taxonomy or microbial ecology. At the same time, it reveals the evolutionary divergence that has accumulated in these genes since the early Proterozoic or the Archaean, and the phylogenetic depth of these metabolisms in the bacterial and archaeal tree of life. With a growing database, homologous and ancestral traits of these genes, including secondary structure motifs and conserved sites, can be found that significantly increase our understanding of environmental and functional constraints that have shaped the evolution of these ancient microbial pathways and enzymes.

However, in spite of continuing primer development (Klein *et al.*, 2001), it is not certain whether PCR-based approaches can reliably detect all environmental genes of interest, in particular the most ancestral and deeply branching lineages or key genes with nonconserved primer sites. Primer site conservation can never be taken for granted. For example, 16S rRNA sequence motifs that were regarded as universally conserved show substantial variation between different bacterial lineages (Daims *et al.*, 1999), and can render 16S rRNA gene amplification with standard primers impossible (Huber *et al.*, 2002). To circumvent primer limitations, surveys of PCR-accessible genes (such as *dsrAB*, *apsA*, and *mrcA*) could be extended by shotgun cloning and fosmid library construction followed by sequence analysis. This approach also addresses the problem of phylogenetic congruence of different marker genes with partially discordant phylogenies. Proving the affiliation of different marker genes to each other and to their host organism requires an extensive database of pure cultures and strains, as shown for the 16S rRNA, *dsrAB* and *apsA* genes in sulfate-reducing prokaryotes, and their partially discordant gene trees (Friedrich, 2002). A genomic solution has to be found if marker genes belong to uncultured lineages in which this ground-truthing approach is not possible. Inferences based on co-occurrence at particular sampling locations with specific geochemical regimes cannot prove that novel phylotypes (for example 16S rRNA and *dsrAB*) belong to the same source organism (Thomson *et al.*, 2001). Tying together such phylotypes in the absence of cultures may require an extensive database of long genomic fragments, in which multiple key genes serve as "phylogenetic anchors" that identify the source organism of a larger genomic fragment and its phylogenetic position in addition to its function (Beja *et al.*, 2000).

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